THE ROLE OF NITRIC OXIDE IN RESOLUTION OF VASOSPASAM CORRESPONDING WITH CEREBRAL VASOSPASMS AFTER SUBARACHNOID HAEMORRHAGE: ANIMAL MODEL

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Abstract

Intracranial aneurysmal rupture is the common cause of spontaneous subarachnoid haemorrhage (SAH). This haemorrhage is typically diffuse and located in extracerebral subarachnoid space in which main cerebral arterial branches are situated. The intimate and long-term contact of arterial wall and blood products in the closed space causes the cerebral vasospasm as a serious and frequent complication of SAH. It is connected with significant morbidity and mortality due to developing of focal cerebral ischaemia and subsequently cerebral infarction. The aim of our experimental research was to create the animal model of vasospasm using the femoral artery due to examination of reduced basic dilator activity cause in arterial wall after SAH. The important characteristic of major cerebral arteries is their localization in the closed subarachnoid space which enables their to have long-term contact with blood products after haemorrhage. Thirty six femoral arteries (FA) of eighteen female rats weighing about 300 g were used. In vivo, femoral arteries are microsurgically prepared in both inguinal regions in all rats. Eighteen arteries were encompassed by polytetrafluoroethylene (PTFE) material forming closed tube and autologous blood was injected in the tube around the arterial wall. Additional eighteen arteries, as a control group, were also put in PTFE tube but without exposing to the blood. All rats are left to live for eight days. Afterwards, rats were sacrificed and their arteries were in vitro examined including an isometric tension measurement and histological changes analysis. The tension was measured during application of vasoconstrictors and vasodilatators (nitric oxide, NO). FA exposed to periadventitial blood exhibit hyper reactivity to constrictors (KCl, phenylephrine, acetylcholine) compared to control group. It was also found that NO donor (sodium nitroprusside) diminished arterial spasm induced by blood and vasoconstrictors. In conclusion, FA can be used as a model for vasospasm correlating with cerebral vasospasm after SAH and therefore this model can be utilized in future experiments assessing cerebral vasospasm. The reduced basic dilator activity of spastic femoral artery is caused by an absence of gaseous messenger NO next to the arteries but not by diminished response vasculature to NO. Absence of NO after SAH probably causes the reduced basic dilator activity of cerebral arteries as well. The guanylate-cyclase level in the arterial wall is consequently reduced after SAH primary due to absence of NO but not due to direct reduction of enzyme activities caused by process of blood degradation inside of subarachnoid space.

KEY WORDS: Familial adenomatous polyposis, microsatellite instability, loss of heterozygosity

Introduction

Cerebral vasospasm is a devastating complication of spontaneous subarachnoid hemorrhage (SAH). The presence of blood products in subarachnoid space cause an imbalance between intrinsic constrictor and dilator mechanisms in cerebral arteries with consecutive increased constrictor mechanism as a crucial contribution for onset and development of vasospasm (1,2,3). Clinical significant and diagnostic measurable vasospasm occurs on approximately day 3 post-SAH, although acute vasospasm is also stressed in novel reports. Recently, nitric oxide is recognized as an important factor in cerebral dilatation, but its exact role in the pathophysiological processes after SAH is not completely elucidated (4,5,6) . The key question associated with post-SAH vasospasm is whether increased basic constrictor activity in arterial wall is caused by reduction of nitric oxide (NO) or by insufficient artery response to NO i.e. by changes of mural arterial enzyme systems in which guanylate (guanylyl)-cyclase plays the most important role (7, 8) If the problem is reduction of NO, the simple NO addition could resolve it. In the case of an impaired vasodilator response of vasculature to NO, the real problem is a reduced capacity of soluble guanylate-cyclase to generate cGMP, which is direct vasodilator (9, 10). Our objectives were to create experimental, animal model of delayed vasospasm using rat femoral artery and to examine the cause of reduced basic dilator activity after SAH with the help of this model.

Materials and Methods

Two hypotheses were put forward concerning this research:

1. A rat femoral artery can be an appropriate model for vasospasm correlating with post SAH spasm in the setting of closed system which implies the long-term contact of blood products with arterial wall and when microsurgical dissection is performed.
2. An impairment of basic vasodilator activity is determined by diminished production of NO (or NO inactivation) but not primary by reduced effectiveness of guanylate-cyclase, enzyme activated by NO.

Eighteen female Wistar rats weighing 290-320 g were used. The thirty-six rat femoral arteries with outer diameter of 0,8 mm (Figure 1.) were in vivo dissected by utilizing microsurgical technique and Zeiss operative microscope. Rats are anesthetized with intraperitoneal Nembutal (35 mg/kg) and allowed to breathe spontaneously. Autologous periadventitial blood was applied to

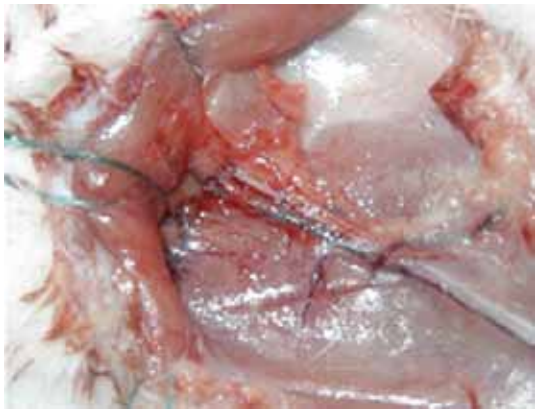

FIGURE 1. The femoral artery of the rat

eighteen femoral arteries and vessels were encompassed by the polytetrafluoroethylene (PTFE) material forming closed tube. Additional eighteen arteries, as a control group, were also put in the PTFE tube but without exposing to the blood. Both groups of arteries in all rats were prepared and left for eight days *in vivo* (Figure 2.). Afterwards all animals were sacrificed and arteries were processed for in vitro examination including an isometric tension measurement and histological changes analysis. The tension was measured in both groups of arteries during application of vasoconstrictors (KCl, phenylephrine, and acetylcholine) and vasodilator – NO. This model corresponds with post -SAH situation of cerebral arteries. The most intensive rat vessels spastic reaction can be noted eight days after exposing arteries to the blood so this period was a right moment for sacrificing animals. Femoral arteries, exposed to the blood and its products during eight days, were excised from the rats as an 8 mm long segment. In vitro this segment was cut in two ring segments of 4 mm length. One segment was sent to the histological analysis. Through lumen of another one, two thin wires were introduced and metal triangles were formed from these wires with the help

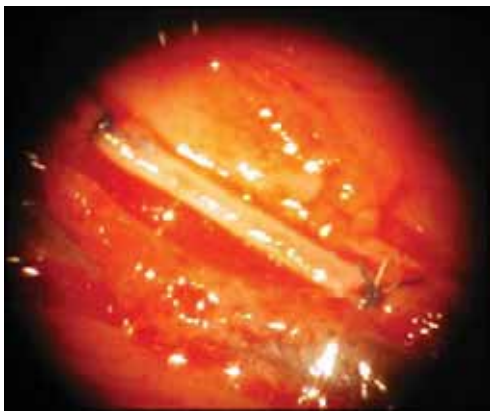

FIGURE 2. Artery in vivo just before excision from PDFE tube

FIGURE 3. Segment of artery in the water bath

of microscope and microinstruments. The metal triangles were connected with nonelastic threads. Arterial rings, prepared in accordance with above-mentioned principles, were brought to the water bath with organ chamber and Krebs's solution due to isometric tension recording. One part of thread was tied to the transducer and another one was fixed in the bath (Figure 3). Vasoconstrictors were added to the both groups of arteries, in the organ chamber, owing to registration of arterial reactivity differences. NO donor (sodium nitroprusside) is also added due to examination of NO modulatory influence to spastic arteries and appraisal of vascular relaxation. Water bath was filled out with distillated water with constant temperature of 37°C. The organ chamber with 20 ml Krebs' solution was situated inside the central part of water bath. Krebs' solution was aerated (95% O_2 i 5% CO_2) under pressure just before the experiment during one and a half hour. The transducer was designed for measuring the forces inside the tunica media of artery. Artery was in isometric tension due to induced vasospasm developed by influence of blood breakdown products. The function of transducer was consistent with the fact that produced forces, brought to carbon fibers, are converted to proportional electrical signal. This signal was visualized and recorded on the millimeter paper by the writer machine. Namely, the writer machine was connected to transducer so arterial tonus with changes in arterial wall was recorded directly. Isometric transducer measures the changes in force in the setting of constant length. We used isometric transducer (7003 model) which was suggested for the forces of 1-3 grams. It is adequate for no sensitive organs or for the small force. Arteries and tracheal rings are the most common organs for examination with this transducer. Krebs' solution was consisted of KH_2PO_4 1,2 mmol/ dm3; KCl 4,7 mmol/ dm3; $CaCl_2$ 2,5 mmol/ dm3; $MgSO_4$ 1,2 mmol/ dm^3; $NaHCO_3$ 25,0 mmol/ dm3; NaCl 118,3 mmol/ dm^3; Dextrose 1,1 mmol/ dm^3. Nitric oxide donor was sodium-nitroprusside/ $Na_2Fe(CN)_5NO$/. The concentration of 1 mmol/ dm3 donor was added to solution. Before donor NO adding the reaction of arteries was examined by using the KCl, phenilephrine and acetylcholine. We used KCL in concentration of 40 mmol/dm^3. Transducer had a system for load adjustment. The loading was made on arterial wall with the help of thread and metal triangle. It caused the extending of arterial wall and its tunica media. After bringing the artery from *in vivo* to *in vitro* conditions, artery was primary put in Krebs' solution (water bath) during 50 min to be adjusted to new conditions. Afterwards, the test length/tension was made. Firstly, artery exposed to blood was examined on the following way:

- Increased the tension on the arterial wall by adjusting the load on the transducer up to 1 gram, and artery was left with that load for 10 minutes.
- Additional load, up to 2 grams, on the arterial wall was made. It was waited for additional 10 minutes.
- Additional load up to 3 grams. Additional 10 minutes waiting
- Adding the 0, 2 ml KCl solution, in the concentration of 4 mol, to the organ chamber which has been filled out with 20 ml of Krebs' solution. On this way we get the adequate concentration of KCl at the organ chamber (40 mmol/ dm3 KCl).
-Washing out and waiting for additional 10 minutes
-Adding of phenylephrine in increasing concentration from 10-7, 10-6 to 10-5 mmol/dm3
-Washing out and leaving the artery at the water bath waiting for additional 15 minutes
-Adding the acetylcholine in the concentration of 10-9 mmol/dm3
-Washing out and waiting for 30 minutes.
-Repetition of whole procedure after primary adding of NO. The same artery was included in the same protocol but with one important difference. Before adding the abovementioned constrictors, artery was treated by sodium nitroprusside (NO donor) in the concentration of 1 mmol/ dm3. In fact, NO was added at the chamber with spastic artery and whole procedure was repeated with KCl, phenylephrine, and acetylcholine.
-Assessment of control group of arteries with the same protocol but without adding NO. The reason for this approach is our intention to compare reaction on the constrictors of two groups of arteries: control group and group of arteries exposed to blood products without NO addition

The group of arteries exposed to blood products and with NO addition should have showed whether NO has an ability to modulate response of arteries in the case when their basic isometric tension are increased. Shortly, we measured the response of arteries on constrictors and that response was recorded on millimeter papers by utilizing the isometric transducer. Transducer was converted the induced forces from the arterial wall in the electrical signal. Fifty minutes after arterial excision, its connection to transducer and fixation to the wall of bath, the addition of abovementioned substances was followed according to described protocol. The response was recorded on millimeter papers. The 4 mm segment of each artery was sent to the histological examination.

Results

Three groups of arteries were created by 36 examined arteries. The first group of arteries included spastic ones e.g. those arteries exposed only to blood (spastic). The second group is the first group which was primary exposed to blood and secondly to the NO donor (spastic + NO). Third group is the control group e. g arteries which were not exposed to blood and NO. Tables 1, 2, and 3 show an arterial response to the constrictors (KCl, phenylephrin, acetylcholine) in the terms of millimeters. For example, the control group showed the minimal response to KCl (in the range 5 to 10 mm) but spastic group showed extensive response (35 to 45 mm). The response was proportional to the tension increase in the arterial wall which means to the strength of contraction (level of spasm) (11, 12). To additionally confirm our results from other point of view, ten spastic arteries were randomized after

Femoral arteries	*1.*	*2.*	*3.*	*4.*	*5.*	*6.*	*7.*	*8.*	*9.*
I (Spastic)	36	40	39	34	42	*41*	38	*39*	39
II (Spastic +NO)	22	25	25	22	26	25	23	23	24
III (Control)	7	8	9	10	9	5	4	8	7
Femoral arteries	*10.*	*11.*	*12.*	*13.*	*14*	*15*	*16*	*17.*	*18.*
I (Spastic)	44	40	36	35	37	35	41	45	39
II (Spastic+ NO)	26	24	22	23	23	22	24	26	24
III (Control)	8	5	9	7	7	10	6	9	6

TABLE 1. Arterial response to KCl in the millimeters.

Femoral arteries	*1.*	*2.*	*3.*	*4.*	*5.*	*6.*	*7.*	*8.*	*9.*
Spastic art.	58	58	59	60	62	*61*	60	*58*	59
Spastic art.+NO	41	41	42	44	44	43	43	40	40
Control art.	12	13	11	19	16	14	11	14	13
Femoral arteries	*10.*	*11.*	*12.*	*13.*	*14*	*15*	*16*	*17.*	*18.*
Spastic art.	62	59	58	61	59	59	62	58	58
Spastic art.+NO	45	43	42	43	41	41	43	41	40
Control art.	17	15	20	17	20	18	15	16	19

TABLE 2. Arterial response to phenylephrinein the millimiteres.

Femoral arteries	*1.*	*2.*	*3.*	*4.*	*5.*	*6.*	*7.*	*8.*	*9.*
Spastic art.	37	39	37	38	34	*32*	33	*35*	36
Spastic art.+NO	21	20	20	20	20	21	22	21	22
Control art.	7	8	8	6	5	5	4	4	7
Femoral arteries	*10.*	*11.*	*12.*	*13.*	*14*	*15*	*16*	*17.*	*18.*
Spastic art.	37	39	32	33	37	37	38	33	32
Spastic art.+NO	22	20	21	20	22	22	21	20	20
Control art.	4	8	7	5	4	4	5	7	6

TABLE 3. Arterial response to acetylcholine in the millimeters.

histological confirmation of morphological changes corresponding with spasm. The Figure 4 illustrate the descent of line on the millimeter papers after adding of NO donor. This means that isometric tension in the arterial wall is decreased. Tension is converted in the electrical signal which is recorded on the papers. The height of one segment of paper is 10 mm.

Arteries	1	2	3	4	5	*6*	7	*8*	9	10
Descending in mm	17	10	18	10	60	18	14	20	20	10

TABLE 4. Descent of line in millimeters as a response of the spastic arteries to NO addition.

Descent of line in mm	Number of arteries
10 mm	3
11mm – 20 mm	6
Over 20 mm	1

TABLE 5. Number of arteries with regard to descent of line in millimeters.

There was a statistically significant difference in descending line on millimeter paper after adding of NO donor meaning there is a significant difference in arterial dilatation i.e. p-value < 0,001 and $X2 = 2,92$. (Table 4 and 5). Histological analysis showed that corrugation of the elastic laminas and endothelial cells changes are constantly present histological findings in spastic arteries (Figure 5).

Discussion

A original rat model for vasospasm which correlates with cerebral vasospasm after SAH has been developed (13, 14). The peripheral artery (15) was microsurgically exposed to autologous blood and situated in closed system in vivo imitating the cerebral artery in subarachnoid space. The contractile responses on KCl, phenylephrine and acetylcholine indicated that arteries exposed to periadventitial blood (spastic arteries) exhibit hyper-reactivity compared to control group of arteries. It was proved that NO modulates spastic arteries reactivity on vasoconstrictor substances. It was also found that previously induced vasospasm can be clearly diminished if sodium nitroprusside (NO donor) is used

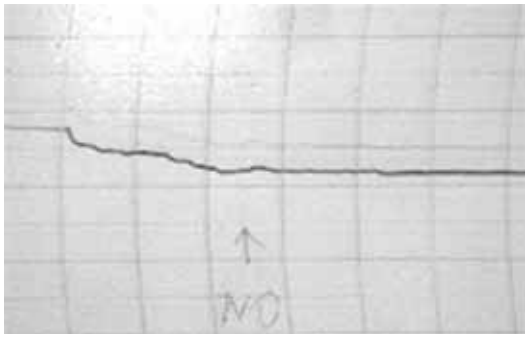

FIGURE 4. Descending line on millimeter paper after adding of NO

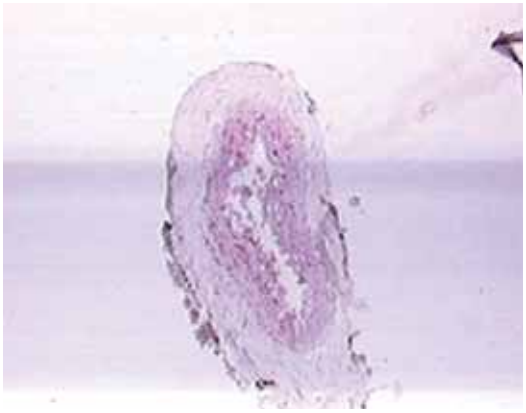

FIGURE 5. Spastic artery

around spastic artery. The nitric oxide increases the basic dilator activities of arterial wall (4, 16, 17). The crucial reason for spasm developing after SAH is absence of NO, not absence of vasculature response to nitric oxide. Absence of NO changes basic tonus of arterial wall which becomes hyper-reactive to constrictors. The autologous periadventitial blood application inside closed PTFE tube containing femoral artery is truly imitation of SAH situation in human, so our model of vasospasm has involved the same mechanisms found in cerebral vasospasm after SAH (1, 3, 16, 18). Additionally, in vitro experiment was also included ten randomized arteries, previously exposed to periadventitial blood in which histological changes corresponding with morphological signs of spasm had already been found. These arteries were added NO donor (sodium nitroprusside). A decrease of isometric tension in arterial wall was noted in the form of descending line on millimeter paper. The tension decrease was a sign of arterial dilatation and a confirmation that even spastic arteries can respond to NO. This was additional argument for hypothesis that post SAH problem is not insufficient artery response to NO due to changes of mural arterial enzyme guanylate (guanylyl)-cyclase which generates cGMP, direct vasodilator. The real problem is reduction of NO itself so the simple NO addition could be a solution. Histological analysis helped us to notice morphological changes in the walls of spastic arteries and to confirm quantitative difference between inner and outer diameter of arteries exposed to blood and those not exposed to blood. The adventitia was not measured because this measurement is uncertain (19). Our animal model of vasospasm with peripheral artery could correspond to other animal model of vasospasm after SAH in which cerebral arteries were used. Our model is simple and can be used easily. Also, the exposing of rat femoral artery is standard part of microsurgical training for neurosurgeons, plastic and vascular surgeons.

Conclusion

1. Femoral artery can be used as a model for vasospasm correlating with cerebral vasospasm after SAH and therefore this model can be utilized in future experiments assessing cerebral vasospasm.
2. The reduced basic dilator activity of spastic femoral artery is caused by an absence of gaseous messenger NO next to the arteries but not by diminished vasculature response to NO.
3. Absence of NO after SAH causes the reduced basic dilator activity of cerebral arteries as well.
4. The cGMP level in the arterial wall is probably reduced after SAH primary due to absence of NO but not due to direct guanylate-cyclase inactivation caused by factors generated from the process of blood degradation inside the subarachnoid space.

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